Interferons activate the p42/44 mitogen-activated protein kinase and JAK-STAT (Janus kinase-signal transducer and activator transcription factor) signalling pathways in hepatocytes: differential regulation by acute ethanol via a protein kinase C-dependent mechanism

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Interferons (IFNs) have been used in the treatment of viral hepatitis. However, their effectiveness is much reduced ($< 10\%$) in alcoholics. The mechanism underlying this resistance remains unknown. Here, we report that IFN-α/β and IFN-γ rapidly activate the JAK-STAT1 (Janus kinase-signal transducer and activator transcription factor 1) and p42/44 mitogen-activated protein kinase (p42/44 MAPK) in freshly isolated rat hepatocytes. Treatment of hepatocytes with 25–100 mM ethanol for 30 min inhibited IFN-β- or IFN-γ-induced STAT1 activation and tyrosine phosphorylation. The inhibitory effect of ethanol was not reversed by pretreatment with either sodium vanadate, a non-selective tyrosine phosphatase inhibitor, or with MG132, a specific proteasome inhibitor. This suggests that protein tyrosine phosphatases or the ubiquitin–proteasome pathway are not involved in the inhibitory action of ethanol. In contrast with the JAK-STAT signalling pathway, acute ethanol exposure significantly potentiated IFN-β or IFN-γ-induced activation of p42/44 MAPK, and caused marked activation of protein kinase C (PKC). Inhibition of PKC partially antagonized ethanol attenuation of IFN-induced STAT1 activation, suggesting that PKC may be involved. Taken together, these findings suggest that the ability of biologically relevant concentrations of ethanol (less than 100 mM) to markedly inhibit IFN-activated STAT1 is one of the cellular mechanisms responsible for the observed resistance of IFN therapy in alcoholics.

Key words: interleukin-6, proteasome, protein-tyrosine phosphatase, tyrosine phosphorylation, viral hepatitis.

INTRODUCTION

Type I (predominantly α/β) and type II (γ) interferons (IFNs) were first identified as inducible secretory proteins with antiviral and antitumor activities, and now have been used in the treatment of a wide variety of diseases [1], including various liver diseases [2–8]. For example, IFN-α/β and IFN-γ have been used in the treatment for viral hepatitis and liver fibrosis [2–8]. The primary form of treatment for viral hepatitis is interferon therapy alone or in combination with Ribavirin. Such therapy can reduce elevated serum alanine transferase levels, eliminate serum virus RNA and improve liver histology [2–8]. Unfortunately, however, less than 10% of alcoholics are responsive to IFN therapy [9–12].

Type I (predominantly α/β) and type II (γ) IFNs signal through distinct but related pathways via binding to type I and type II receptors respectively. The binding of IFNs to their receptors activates the receptor-associated tyrosine kinases [JAK1 and TyK2 associate with the two chains of IFN-α/β receptor, JAK1 (Janus kinase 1) and JAK2 with the IFN-γ receptor chains]. This receptor–kinase complex interacts with, and activates, the SH2-containing cytoplasmic STATs (signal transducer and activator transcription factors) (IFN-α/β activates STAT1, STAT2 or STAT3; IFN-γ activates STAT1, STAT3 or STAT5, depending on cell type). Activated STAT proteins then form homo- or hetero-complexes that translocate to the nucleus to activate the transcription of many target genes (reviewed in [13–15]). In addition to the JAK-STAT signalling pathway, IFNs also activate p42/44 mitogen-activated protein kinase (p42/44 MAPK) [16–18] and insulin receptor substrate-1 (IRS1) [19,20]. The role of IFN-activated STAT1 in antiviral and antitumour activities is clearly demonstrated in STAT1 knockout mice [21,22]. In these mice, IFN signalling is defective and the innate response to viral or bacterial infection is absent. Furthermore, STAT1-deficient mice as well as IFN-γ-deficient mice have a higher incidence of tumours [21,22]. However, the roles of p42/44 MAPK and IRS1 in the function of IFNs are not clear.

Although IFNs have been widely used in the treatment of liver diseases, the IFN signalling pathway in the liver and the mechanisms for unresponsiveness to IFNs in alcoholics remain unknown. In the present study we demonstrate that IFNs activated both p42/44 MAPK and JAK-STAT signalling pathways in freshly isolated hepatocytes. These signalling pathways are differentially regulated by acute ethanol treatment. The ability of biologically relevant concentrations (less than 100 mM) of ethanol to inhibit IFN-activated STAT1 may, at least in part, account for the observed resistance of IFN therapy in alcoholic patients [9–12].

MATERIALS AND METHODS

Materials

STAT1, STAT3, and STAT5 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-

Abbreviations used: JAK-STAT, Janus kinase-signal transducer and activator transcription factor; p42/44 MAPK, p42/44 mitogen-activated protein kinase; PKB/Akt, protein kinase B; IFN, interferon; IL-6, interleukin-6; IRS1, insulin receptor substrate-1; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; DMSA, DNA gel-mobility-shift assay; SH, Src homology.

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phospho-STAT1 (Tyr^{701}), anti-phospho-p42/44 MAPK (Tyr^{204}), anti-[phospho-c-Jun N-terminal kinase (JNK)] (Thr^{183}/Tyr^{185}), anti-phospho-Akt (Ser^{473}) antibodies were obtained from Bio-lab (Beverly, MA, U.S.A.). The following reagents were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.): ethanol, collagenase type IV, sodium vanadate, Nonidet P40. GF- 109203X, and MG132 were obtained from Calbiochem (San Diego, CA, U.S.A.). IFN-α, IFN-β and IFN-γ were purchased from Bioscience International (Camarillo, CA, U.S.A.). Radiolabelled [γ-^{32}P]ATP was purchased from DuPont NEN (Boston, MA, U.S.A.).

Isolation of hepatocytes

Liver cells were isolated by a collagenase-perfusion protocol as described previously [23]. Isolated cells were washed twice and resuspended in Krebs–Henseleit solution (118 mM NaCl/4.7 mM KCl/1.2 mM $MgSO_4$/2.5 mM $CaCl_2$/1.2 mM KH_2PO_4/25 mM $NaHCO_3$/10 mM glucose) containing 1.5 % gelatin, and were further treated with drugs and/or ethanol and/or IFN.

DNA gel-mobility-shift assay (DMSA)

DMSA for STAT binding was described previously [24,25]. The STAT binding site of oligo m67 (the high-affinity serum-inducible element m67) (5′-GTCGACATTTCCCGTAAATCGTCGA-3′) was used as a probe.

Western-blot analysis

Western-blot analysis was described previously [23]. Hepatocytes were re-suspended in lysis buffer [30 mM Tris (pH 7.5)/150 mM NaCl/1 mM PMSF/1 mM Na_3VO_4/1 % Nonidet P40/10 % glycerol] and then centrifuged for 10 min at 4 °C. The protein concentration of the supernatant (protein fraction) was calculated using the Bio-Rad protein assay. An aliquot of 40 μg of protein was mixed with an equivalent volume of 2 × protein loading buffer, pH 7.4, containing β-mercaptoethanol and boiled for 5 min before loading on to an SDS/8 %-polyacrylamide gel. After electrophoresis, proteins were transferred on to nitrocellulose membranes and blotted against primary antibodies. Membranes were washed with TPBS (0.05 % Tween 20 in PBS, pH 7.4) and incubated with a 1:4000 dilution of horseradish peroxidase-conjugated secondary antibodies for 45 min. Protein bands were revealed by an enhanced-chemiluminescence (ECL*) reaction (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

P42/44 MAPK, JNK and protein kinase B (PKB/Akt) phosphorylation

P42/44 MAPK, JNK, and PKB/Akt phosphorylation were detected by Western-blot analysis using specific anti-phospho-p42/44 MAPK (Tyr^{204}), anti-phospho-JNK (Thr^{183}/Tyr^{185}), and anti-phospho-PKB/Akt (Ser^{473}) antibodies respectively.

Protein kinase C (PKC) assay

PKC activity was determined by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to histone IIIs as described previously [26]. Briefly, the reaction was initiated by the addition of 50 μg of protein from the cytosol or membrane fraction to a reaction mixture (0.25 ml volume) containing 20 mM Tris/HCl, 0.75 mM $CaCl_2$, 20 mM magnesium acetate, 5 μg of leupeptin, 100 μg of histone IIIs, 100 μM (60 c.p.m./pmol) [γ-^{32}P]ATP at pH 7.5, in the absence or presence of 25 μg of phosphatidylserine (PKC cofactor) and 1 μg of diacylglycerol (PKC activator). Samples were incubated for 5 min at 31 °C and the reaction was terminated by the addition of 1 ml of 25 % (v/v) trichloroacetic acid. Tubes were centrifuged at 5000 ***g*** for 15 min and the pellet washed three times with 4 ml of cold trichloroacetic acid (10 %, v/v). Precipitates were solubilized with 0.5 ml of 2 M NaOH and transferred to vials for liquid-scintillation counting. PKC activity was calculated by subtracting the amount of ^{32}P incorporated in the absence of phospholipids (non-PKC) from the amount of ^{32}P incorporated in the presence of phospholipids, and this was expressed as pmol of ^{32}P incorporated into histone III/min per mg of protein at 31 °C. Enzyme activity was observed to be linear up to 15 min. The cytosol and membrane fractions were prepared as described previously [26]. Briefly, hepatocytes were resuspended in 1 ml of homogenization buffer A (20 mM Tris/HCl/330 mM sucrose/2 mM EDTA/0.5 mM EGTA/2 mM PMSF, pH 7.5), homogenized with 30 strokes on ice and then centrifuged at 100000 ***g*** for 1 h. The supernatant was used as the cytosol fraction. The remaining pellet was resuspended in 1 ml of buffer A containing 1 % Nonidet P40 and homogenized for ten strokes, incubated on ice for 30 min and then centrifuged at 100000 ***g*** for 15 min. The supernatant was used as the membrane fraction.

RESULTS

IFN-α, IFN-β and IFN-γ activate the JAK-STAT1 signalling pathway in freshly isolated hepatocytes

As shown in Figure 1(A), exposure of freshly isolated hepatocytes to IFN-α, IFN-β, or IFN-γ for 20–50 min led to a significant increase in STAT binding to the specific m67 oligonucleotide probe (lanes 1–4, lanes 5–8 and lanes 9–12). To identify the STAT protein that was involved, a DNA gel supershift assay was performed (Figure 1B). As shown in Figure 1(B), STAT1 antibody, but not STAT3 or STAT5 antibody, was able to supershift the IFN-β-activated complex. Similar results were obtained with IFN-γ-activated complex (results not shown). These findings suggest that IFNs activate only STAT1, but not STAT3 or STAT5, in primary rat hepatocytes. Moreover, Western-blotting analysis in Figure 1(C) showed that IFN-β or IFN-γ activated STAT1, but not STAT3, tyrosine phosphorylation, while interleukin-6 (IL-6) induced both STAT1 and STAT3 tyrosine phosphorylation (lane 4). Furthermore, IFN-α, IFN-β and IFN-γ also activated STAT1 in human hepatocellular carcinoma HepG2 cells (results not shown). These findings indicate that IFN-α, IFN-β and IFN-γ activate the JAK-STAT1 signalling pathway in freshly isolated hepatocytes and HepG2 cells. Since IFN-α and IFN-β both act on the similar type I receptor [13–15], we only studied the effects of ethanol on one of the representative IFN-β signalling pathways described in the present paper.

Effects of IFN-α, IFN-β, and IFN-γ on p42/44 MAPK, PKB/Akt and p46/54 JNK in freshly isolated hepatocytes

As shown in Figure 2(A), treatment of freshly isolated hepatocytes with either IFN-α or IFN-β rapidly activated p42/44 MAPK, the peak effect occurring at 10 and 20 min respectively, and declined towards control levels after 60 min exposure. IFN-γ treatment also activated p42/44 MAPK in hepatocytes, with the peak activation at 5 min and declining towards control levels after 20 min treatment. Interestingly, upon IFN-γ incubation for 60 min, p42/44 MAPK phosphorylation increased again. Activation of p42/44 MAPK by IFN-α/β or IFN-γ was also observed in HepG2 cells (results not shown). Positive control

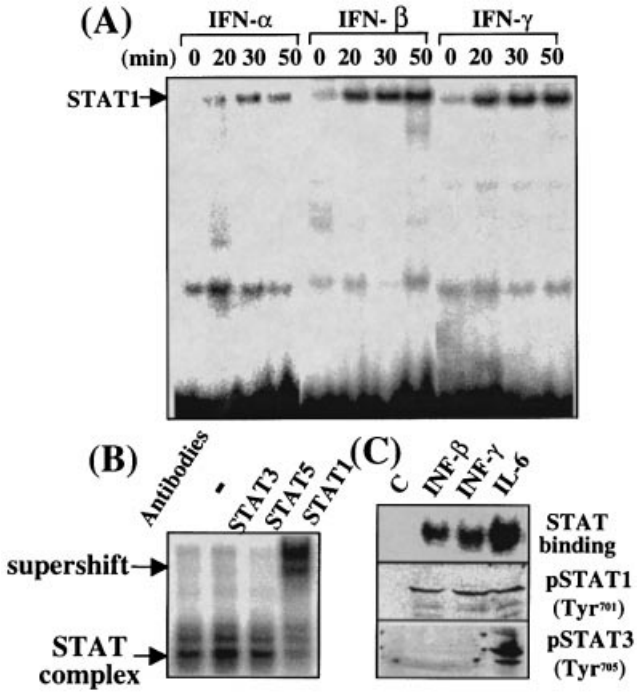

Figure 1 IFNs activate STAT1 in primary hepatocytes

(**A**) Freshly isolated hepatocytes were incubated with IFN-α (250 units/ml), IFN-β (250 units/ml) or IFN-γ (10 ng/ml) for various time periods as indicated. Whole-cell extracts were prepared and DMSA was performed using the m67 probe, as described in the Materials and methods section. (**B**) Hepatocytes were stimulated with IFN-β (250 units/ml) for 30 min. Whole-cell extracts were incubated with various STAT antibodies as indicated and subjected to a supershift assay. (**C**) Hepatocytes were stimulated with IFN-β ('INF-β') (250 units/ml), IFN-γ ('INF-γ') (10 ng/ml) or IL-6 (20 ng/ml) for 30 min. Whole-cell extracts were subjected to DMSA to examine STAT binding or Western-blotting analysis using anti-phosphotyrosine STAT1 (Tyr^{701}) or anti-phosphotyrosine STAT3 (Tyr^{705}). An autoradiogram representative of three independent experiments is shown in each panel.

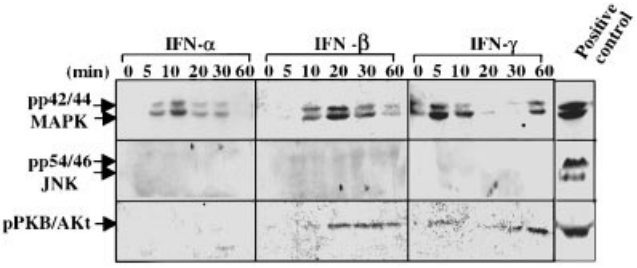

Figure 2 Effects of IFNs on p42/44 MAPK, PKB/Akt and JNK in primary hepatocytes

Freshly isolated hepatocytes were incubated with IFN-α (250 units/ml), IFN-β (250 units/ml) or IFN-γ (10 ng/ml) for various time periods as indicated. Whole-cell extracts were subjected to Western-blotting analysis using anti-phospho-p42/44 MAPK, anti-phospho-JNK or anti-phospho-PKB/Akt antibodies as indicated. Hepatocytes were stimulated with EGF (10 ng/nl), tumour necrosis factor-α (20 ng/ml) and phenylephrine (10^{-5} M) for 10 min as positive controls for p42/44 MAPK, JNK and PKB/Akt respectively. An autoradiogram representative of three independent experiments is shown in each panel.

activation of p42/44 MAPK by epidermal growth factor (EGF) was 5-fold stronger than that observed with IFN.

Effects of IFNs on PKB/Akt and JNK were also examined. As shown in Figure 2, neither IFN-α, nor IFN-β and IFN-γ,

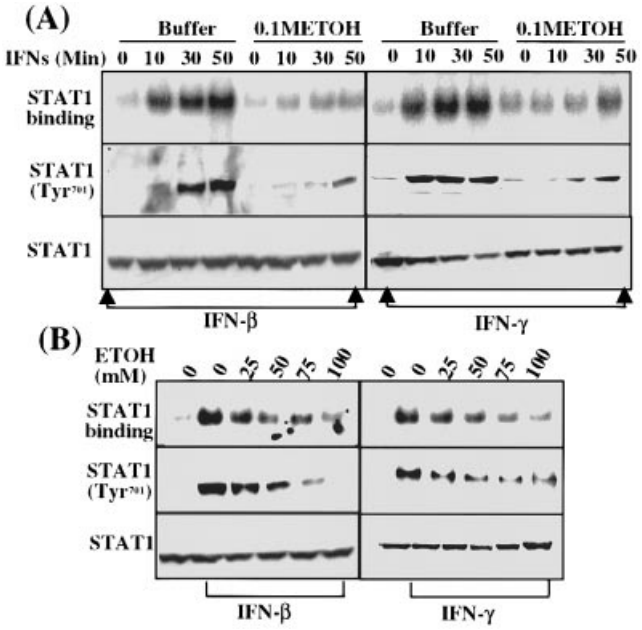

Figure 3 Acute ethanol inhibits IFN-induced STAT1 binding and tyrosine phosphorylation

(**A**) Freshly isolated rat hepatocytes were incubated with 100 mM ethanol or buffer for 30 min, then stimulated with IFN-β (250 units/ml) or IFN-γ (10 ng/ml) for various time periods as indicated. (**B**) Freshly isolated hepatocytes were incubated with various concentrations of ethanol for 30 min, followed by a 30 min stimulation with IFN-β (250 units/ml) or IFN-γ (10 ng/ml). In (**A**) and (**B**), after treatment, whole-cell extracts were prepared and subjected to DMSA to examine STAT binding or Western-blotting analysis using anti-phosphotyrosine STAT1 (Tyr^{701}) or anti-STAT1 antibodies. An autoradiogram representative of three independent experiments is shown in each panel.

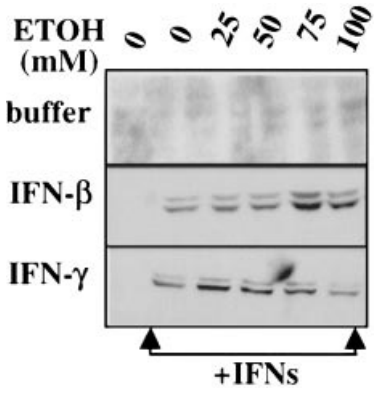

Figure 4 Acute ethanol potentiates IFN-activated p42/44 MAPK

Freshly isolated rat hepatocytes were pretreated with various concentrations of ethanol, followed by a 10 min stimulation with buffer, IFN-β (250 units/ml) or IFN-γ (10 ng/ml). Whole-cell extracts were then subjected to Western-blotting analysis using an anti-phospho-p42/44 MAPK antibody. The autoradiograms shown are representative of three independent experiments.

activated p46/54 JNK, whereas positive control tumour necrosis factor-α significantly activated this kinase. IFN-β or IFN-γ, but not IFN-α, also slightly activated PKB/Akt in primary hepatocytes. Positive control phenylephrine markedly stimulated this kinase. Since IFNs did not significantly activate JNK and weakly activated PKB/Akt, effects of ethanol on these two kinases were not further studied here.

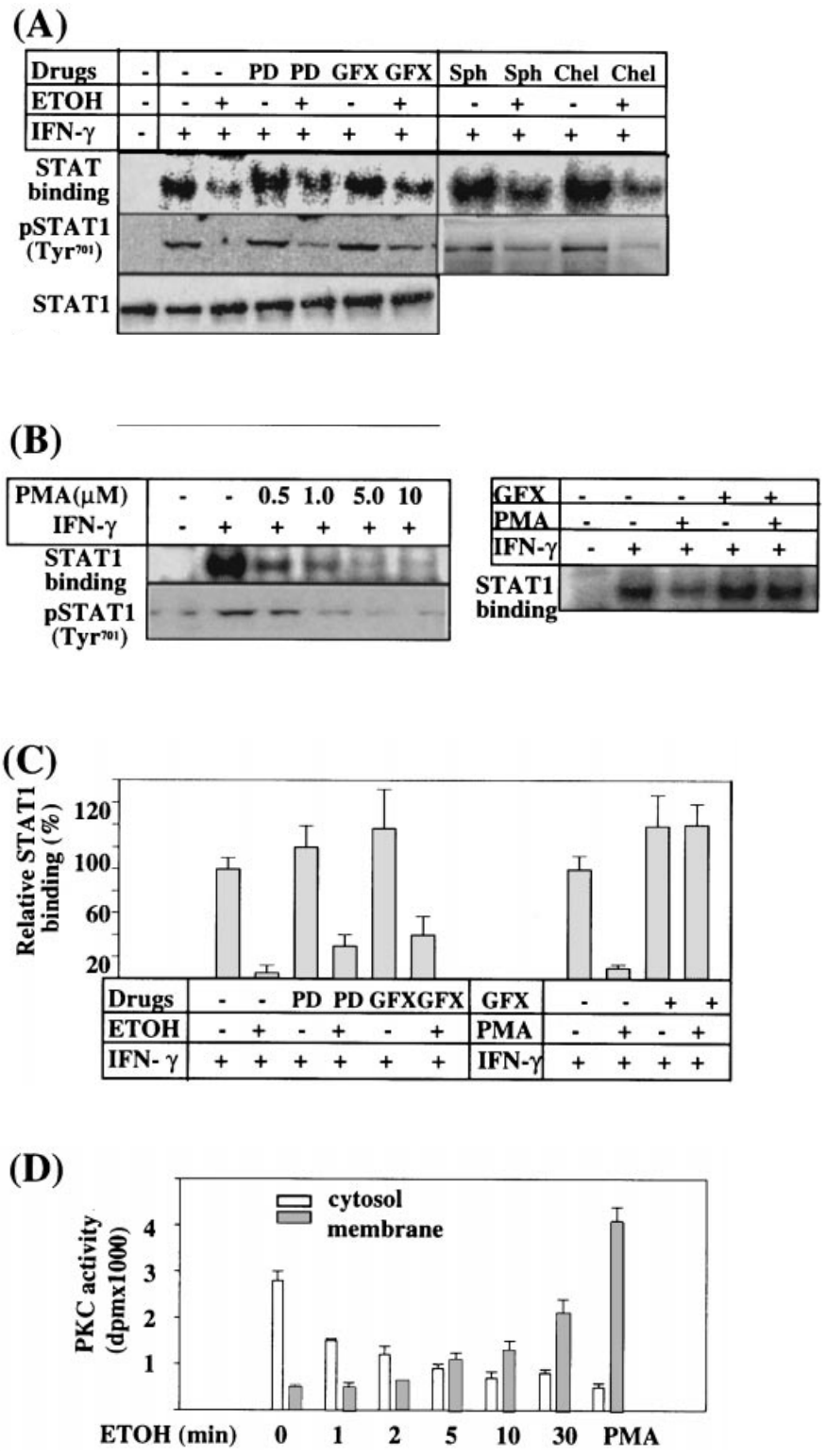

Figure 5 Evidence for the involvement of PKC in ethanol suppression of IFN-induced STAT1

(**A**) Freshly isolated rat hepatocytes were incubated with 50 μM PD98059 (PD), 2 μM GF109203X (GFX), 1 μM sphingosine (Sph) or 0.5 μM chelerythrine (Chel) for 30 min, then with 100 mM ethanol for 30 min, followed by a 30 min stimulation with IFN-γ (10 ng/ml) for 30 min. Cell extracts were then subjected to DMSA to detect STAT1 binding or Western-blot analysis using anti-phospho-STAT1 (Tyr^{701}) or anti-STAT1 antibodies. (**B**) In left panel, freshly isolated rat hepatocytes were incubated with PMA (0.5–10 μM) for 30 min, followed by a 30 min stimulation with IFNγ (10 ng/ml). In the right panel, freshly isolated hepatocytes were incubated with 2 μM GF109203X for 30 min, then with 0.5 μM PMA for 30 min, followed by a 30-min stimulation with IFNγ (10 ng/ml). Cell extracts in both left and right panels were subjected to DMSA to detect STAT1 binding. (**A**) and (**B**) are representative of three to seven independent experiments. (**C**). The autoradiograms were quantified by phosphorimaging from (**A**) and (**B**). Values shown are means $\pm$ S.E.M. for three to seven independent experiments, expressed as fold changes over control. (**D**) Freshly isolated hepatocytes were incubated with 100 mM ethanol for various time periods as indicated or with 1 μM PMA for 10 min. Cytosol and membrane protein fractions were prepared and subjected to the PKC assay described in the Materials and methods section.

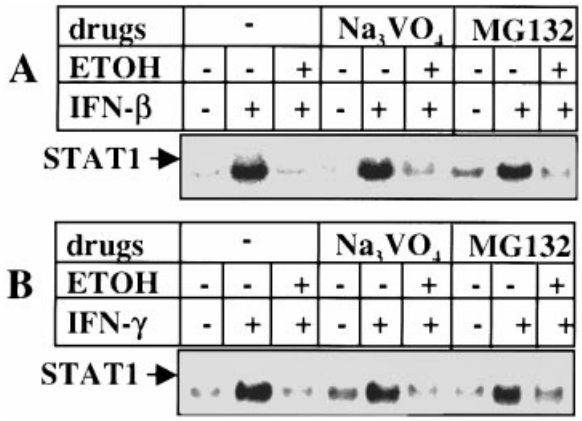

Figure 6 Tyrosine phosphatases and the ubiquitin–proteasome pathway are not involved in ethanol suppression of IFN-induced STAT1 activation

Freshly isolated rat hepatocytes were pretreated with buffer, sodium vanadate (0.2 mM) or proteasome inhibitor (50 μM MG132) for 30 min, followed by incubation with or without 100 mM ethanol for 30 min, and then stimulated with 250 units/ml IFN-β (**A**) or 10 ng/ml IFN-γ (**B**) for 30 min. DMSA was performed using the m67 probe. An autoradiogram representative of three independent experiments is shown in each panel.

Acute ethanol inhibits IFN-β- or IFN-γ-induced STAT1 activation and tyrosine phosphorylation

To examine the effects of ethanol on IFN-induced STAT1 activation, freshly isolated hepatocytes were incubated with 100 mM ethanol for 30 min, followed by stimulation with IFNs for various time periods. As shown in Figure 3(A), 100 mM ethanol almost completely abolished IFN-β- or IFN-γ-induced STAT1 binding to the m67 probe. Furthermore, IFN-β- or IFN-γ-induced STAT1 activation was inhibited by a 30 min pretreatment with ethanol in a concentration-dependent manner, with inhibition evident at 25 mM and complete inhibition at 100 mM ethanol (Figure 3B).

To examine whether rapid inhibition of IFN-β- or IFN-γ-induced STAT1 activation by ethanol was due to suppression of STAT1 tyrosine phosphorylation, Western-blot analysis was performed using an anti-phospho-STAT1 (Tyr^{701}) antibody. As shown in Figure 3(A), it was observed that either IFN-β or IFN-γ induced significant STAT1 tyrosine phosphorylation, and pretreatment of hepatocytes with 100 mM ethanol for 30 min abolished this activation. Ethanol inhibition of IFN-induced STAT1 tyrosine phosphorylation was also concentration-dependent, with inhibition evident at 25 mM (Figure 3B). Ethanol alone did not affect basal STAT1 tyrosine phosphorylation (results not shown). As illustrated in the bottom panels of Figures 3(A) and 3(B), the same ethanol treatment did not significantly affect the cellular level of the STAT1 protein expression. These findings suggest that acute exposure of hepatocytes to biologically relevant concentrations of ethanol markedly inhibits IFN-activated STAT1. The inhibitory effect of ethanol on IFN-activated STAT1 is not non-specific, since the same ethanol treatment potentiated IFN-activated p42/44 MAPK (see below).

Acute ethanol potentiates IFN-β- and IFN-γ-activated p42/44 MAPK

Effect of ethanol on p42/44 MAPK was examined. As shown in Figure 4(A), exposure of primary hepatocytes to various concentrations of ethanol for 30 min did not significantly affect the basal levels of p42/44 MAPK (top panel), but significantly potentiated IFN-β- and IFN-γ-activated p42/44 MAPK. Activation was most evident at 50–75 mM ethanol.

Activation of PKC is partially involved in ethanol inhibition of IFN-activated STAT1

Activation of PKC has been proposed to mediate several effects of ethanol, such as potentiation of p42/44 MAPK [27] or desensitization of receptor-mediated phospholipase C activation [28]. To test whether activation of PKC was involved in ethanol inhibition of IFN-activated STAT1, a highly specific PKC inhibitor, GF109203X, was used. As shown in Figure 5(A), blocking PKC activation with 2 μM GF109203X partially prevented ethanol inhibition of IFN-γ-induced STAT1. In this experiment, as well as seven replicate experiments with similar results, GF109203X partially (30%) antagonized ethanol suppression of IFN-γ-activated STAT1 (Figure 5C). Other PKC inhibitors, such as sphingosine and chelerythrine, also partially antagonized ethanol suppression of IFN-γ-activated STAT1 (Figure 5A). Similar experiments with IFN-α/β and IL-6 were conducted and suggest that PKC is also partially involved in ethanol inhibition of IL-6-activated STAT3 and IFN-α/β-activated STAT1 (results not shown). Moreover, PD98059, a specific MEK1 inhibitor, also partially reversed ethanol inhibition of IFN-activated STAT1 (Figure 5A), suggesting that activation of p42/44 MAPK is partially involved.

To examine further the role of PKC in ethanol suppression of STAT activation, two experiments were carried out. First, we investigated whether the PKC activator PMA was able to inhibit STAT activation induced by either IFNs or IL-6. As shown in Figures 5(B) and 5(C), pretreatment of primary hepatoctyes with 0.5 to 10.0 μM PMA significantly inhibited IFN-γ-activated STAT1 binding and tyrosine phosphorylation. GF109203X, a highly selective PKC inhibitor, completely abolished PMA inhibition of STAT activation (Figures 5B and 5C). Similar experiments with IFN-α/β and IL-6 demonstrated that PMA attenuated IL-6-activated STAT3 and IFN-α/β-activated STAT1 (results not shown).

Secondly, we examined whether ethanol was able to induce PKC activation and membrane translocation. Freshly isolated hepatocytes were incubated with 100 mM ethanol for various time periods. Cytosol and membrane fractions of cell lysates were prepared and examined for PKC enzymic activity. Incubation of freshly isolated hepaotcytes with ethanol decreased PKC activity in the cytosol fraction and increased it in the membrane fraction (Figure 5D), suggesting that ethanol stimulates PKC translocation from cytosol to membrane after activation. Western-blotting analysis also showed that acute exposure of freshly isolated hepatocyte to ethanol slightly caused PKCα and PKCβ1 protein translocation from cytosol to membrane (results not shown), further supporting the notion that acute ethanol exposure slightly activates PKC.

Tyrosine phosphatases and the ubiquintin–proteasome pathway are not involved in ethanol suppression of IFN-induced STAT1 activation

The data from Figure 5 demonstrated that GF109203X completely antagonized PMA inhibition of STAT activation, but only partially (30%) attenuated ethanol inhibition of STAT activation. This suggested that PKC could not fully explain the inhibitory action of ethanol. Thus the next obvious question was

what other mechanisms are involved in ethanol suppression of STAT activation. Both tyrosine phosphatases and the ubiquitin–proteasome pathway have been implicated in inactivation of the JAK-STAT signalling pathway (reviewed in [29–31]); we wondered whether these pathways were involved in ethanol inhibition of IFN-activated STAT1. To test these possibilities, the non-selective tyrosine phosphatase inhibitor sodium orthovanadate and the specific proteasome inhibitor MG132 were used. As shown in Figures 6(A) and 6(B), neither sodium orthovanadate nor MG132 pretreatment antagonized ethanol suppression of IFN-β- or IFN-γ-activated STAT1, while the same treatment markedly reversed IL-1β suppression of IFN-α-activated STAT1 (X. Shen, Z. Tian, and B. Gao, unpublished work). This suggests that both tyrosine phosphatases and the proteasome-dependent pathway are not involved in ethanol suppression of IFN-induced STAT1 activation.

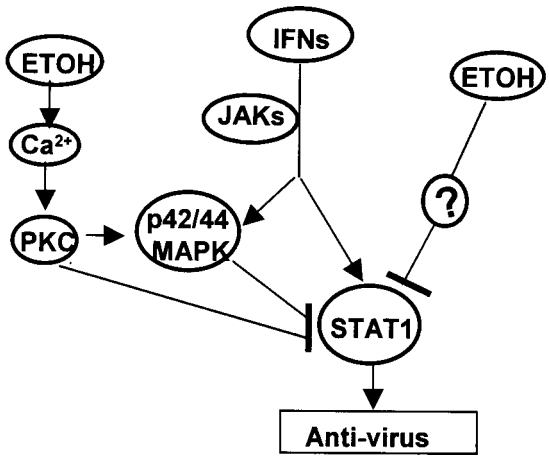

Figure 7 Model for ethanol suppression of IFN-induced STAT1 activation

IFN activates both p42/44 MAPK and STAT1 signalling pathways in primary hepatocytes, and activation of p42/44 MAPK negatively regulates STAT1 activation. Acute exposure of hepatocytes to ethanol induces a rapid rise in cytosolic Ca^{2+} [28] and a consequent activation of PKC. Activated PKC can directly attenuate IFN-activated STAT1 or indirectly inhibit it by potentiation of IFN-activated p42/44 MAPK. Other unknown mechanisms (?) may also be involved in ethanol suppression of IFN-activated STAT1 in freshly isolated hepatocytes.

DISCUSSION

IFNs have been shown to activate various signalling pathways in many cell types [13–15], but their signalling pathways in primary hepatocytes have not been explored. Here we demonstrate that both IFN-α/β and IFN-γ are able to activate the JAK-STAT1, p42/44 MAPK, and PKB/Akt in rat primary hepatocytes. IFN-γ has been shown to activate specific STAT1, STAT3 and STAT5 in certain cell types. For example, IFN-γ activates only STAT1 in some cell types [32,33], both STAT1 and STAT3 in adipocytes [34], and only STAT5 in U937 cells [35]. Here we show that IFN-γ and IFN-β activate only STAT1 in rat primary hepatocytes (Figure 1). The inability of IFNs to activate STAT3 and STAT5 in hepatocytes could be due to difference in the receptor expression, binding affinities, the presence or absence of other signalling proteins, or STAT dimer composition.

It has been reported that IFNs are able to activate the p42/44 MAPK signalling pathway in certain cells, and here we demonstrate that both IFN-α/β and IFN-γ activate p42/44 MAPK, but not JNK, in primary hepatocytes (Figure 2) and in human hepatoma HepG2 cells (results not shown). Compared with EGF, IFNs are weak activators of p42/44 MAPK, as shown in Figure 2, where EGF activation of p42/44 MAPK was 5-fold stronger than IFN. The connection between the JAK-STAT1 and the p42/44 MAPK signalling pathways is not clear. It has been shown that activation of p42/44 MAPK can specifically phosphorylate STAT3 on Ser^{727} in response to growth factor [36] and negatively regulate STAT3 activation [24,37]. However, STAT1 appears to be a poor substrate for all MAPK superfamily members [36], and the kinase responsible for STAT1 Ser^{727} phosphorylation has not been identified. Here we demonstrated that blocking p42/44 MAPK activation by PD98059 slightly potentiated IFN-γ-induced STAT1 binding and STAT1 Tyr^{701} phosphorylation (Figure 5), suggesting that activation of p42/44 MAPK may antagonize IFN-γ-induced STAT1 activation. The role of p42/44 MAPK in the inactivation of STATs is not clear and requires further studies.

An interesting finding in the present study is that, in freshly isolated rat hepatocytes, biologically relevant concentrations (less than 100 mM) of ethanol rapidly and dose-dependently inhibited IFN-β- or IFN-γ-induced activation of STAT1, but also potentiated IFN-β or IFN-γ-induced activation of p42/44 MAPK. Three lines of evidence suggest that activation of PKC is involved in this event. First, the specific PKC inhibitor GF109203X caused marked attenuation of ethanol suppression of IFN-induced STAT1 activation (Figures 5A and 5C). Secondly, activation of PKC by PMA blocked IFN-induced STAT1 activation (Figures 5B and 5C). Thirdly, acute ethanol treatment caused rapid and marked activation of PKC in primary hepatocytes (Figure 5D). Furthermore, Figures 5(A) and 5(C) showed that inhibition of p42/44 MAPK activation by PD 98059 only slightly potentiated IFN-activated STAT1 and partially antagonized ethanol inhibition of IFN-activated STAT1. This suggested that p42/44 MAPK may also be involved in the inhibitory effect of ethanol on STAT activation.

Interestingly, our (J. Chen and B. Gao) unpublished work demonstrated that biologically relevant concentrations of ethanol attenuated STAT activation in freshly isolated hepatocytes, but not in cultured hepatocytes or hepatocullular carcinoma HepG2 cells. Further experiments indicated that the lack of inhibitory effect of ethanol on cultured hepatocytes and HepG2 cells was not due to ethanol metabolism or proliferation status. These findings suggest that freshly isolated hepatocytes are more sensitive to ethanol inhibition of the JAK-STAT signalling pathway than cultured hepatocytes or HepG2 cells, which may be implicated in pathogenesis and progression of alcoholic liver diseases.

Inhibition of PKC by GF109203X completely abolished PMA attenuation of IFN-γ-activated STAT1 (Figures 5B and 5C), but only partially (about 30%) blocked ethanol inhibition of IFN-activated STAT1 (Figures 5B and 5C) or IL-6-activated STAT3 (results not shown). These findings suggest that PKC is only partially involved in, and cannot fully account for, ethanol suppression of STAT activation. Several mechanisms responsible for inactivation of the JAK-STAT signalling pathway have been proposed (reviewed in [29–31]. For example, activated JAK-STAT can been attenuated by: (1) dephosphorylation by protein tyrosine phosphatases; (2) proteolytic degradation by the ubiquitin–proteasome pathway; (3) inhibitory molecules such as SOCS/JAB/SSI/CIS. In the present study the lack of effect of sodium vanadate and MG132 on the inhibitory action of ethanol on STAT1 activation suggests that protein tyrosine phosphatases and the ubiquitin–proteasome pathway are not involved. The inhibitory molecules are also unlikely to be involved, because

these proteins are newly synthesized after stimulation with IFN, whereas ethanol inhibitory action is rapid and does not require new protein synthesis (X. Shen, Z. Tian and B. Gao, unpublished work). Thus the molecular mechanisms responsible for ethanol inhibition of STAT activation are not fully understood and require further investigation.

Taken together, we have integrated each of our findings with a proposed model (summarized in Figure 7) that allows for cross-talk between ethanol, IFN-activated p42/44 MAPK and STAT1. In this model, acute exposure of hepatocytes to ethanol induces a rapid but transient activation of phosphoinositide-specific phospholipase C and a consequent rise in cytosolic Ca^{2+} and diacylglycerol [28], which can activate PKC. Activated PKC can directly attenuate IFN-activated STAT1 or indirectly inhibit it by potentiation of IFN-activated p42/44 MAPK. Ethanol may also inhibit IFN-activated STAT1 by some unknown mechanisms. We have previously shown that acute ethanol rapidly and significantly inhibited IL-6-activated STAT3 in freshly isolated hepatocytes [38], suggesting that ethanol may interfere with the phosphorylation of STAT protein in general and its molecular target may be conserved domains of STAT proteins, such as the Src homology 2 (SH2) domain. Indeed, it has been reported that several kinases containing SH2 domains can be affected by ethanol [39–42]. In view of the critical role of IFN-activated STAT1 as a protective agent in viral infection [21,22], ethanol suppression of IFN-activated STAT1 may, at least in part, account for the high incidence of viral hepatitis and unresponsiveness to IFN therapy in alcoholics.

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REFERENCES

1 De Maeyer, E. and De Maeyer-Guignard, J. (1998) Interferons. In Cytokine Handbook (Thomson, A., ed.), pp. 491–516, Academic Press, San Diego
2 Davis, G. L. (1999) Combination therapy with interferon-α and ribavirin as retreatment of interferon relapse in chronic hepatitis C. Semin. Liver Dis. **19**, 49–55
3 Younossi, Z. M. and Perrillo, R. P. (1999) The roles of amantadine, rimantadine, ursodeoxycholic acid, and NSAIDs, alone or in combination with α interferons, in the treatment of chronic hepatitis C. Semin. Liver Dis. **19**, 95–102
4 Gish, R. G. (1999) Standards of treatment in chronic hepatitis C. Semin. Liver Dis. **19**, 35–47
5 Lindsay, K. L. (1997) Therapy of hepatitis C: overview. Hepatology **26**, 71S–77S
6 Keeffe, E. .B. and Hollinger, F. B. (1997) Therapy of hepatitis C: consensus interferon trials: Consensus Interferon Study Group. Hepatology **26**, 101S–107S
7 Zein, N. N. (1998) Interferons in the management of viral hepatitis. Cytokines Cell. Mol. Ther. **4**, 229–241
8 Christie, J. M. and Chapman, R. W. (1999) Combination therapy for chronic hepatitis C: interferon and ribavirin. Hosp. Med. **60**, 357–361
9 Schiff, E. R. (1999) The alcoholic patient with hepatitis C virus infection. Hepatology **26**, 39S–42S
10 Ohnishi, K., Matsuo, S., Matsutani, K., Itahashi, M., Kakihara, K., Suzuki, K., Ito, S. and Fujiwara, K. (1996) Interferon therapy for chronic hepatitis C in habitual drinkers: comparison with chronic hepatitis C in infrequent drinkers. Am. J. Gastroenterol. **91**, 1374–1379
11 Okazaki, T., Yoshihara, H., Suzuki, K., Yamada, Y., Tsujimura, T., Kawano, K., Yamada, Y. and Abe, H. (1994) Efficacy of interferon therapy in patients with chronic hepatitis C. Comparison between non-drinkers and drinkers. Scand. J. Gastroenterol. **29**, 1039–1043
12 Mochida, S., Ohnishi, K., Matsuo, S., Kakihara, K. and Fujiwara, K. (1996) Effect of alcohol intake on the efficacy of interferon therapy in patients with chronic hepatitis C as evaluated by multivariate logistic regression analysis. Alcohol Clin. Exp. Res. **20**, 371–377
13 Stark, G. R., Kerr, I. M., Willams, B. R. G., Silverman, R. H. and Schreiber, R. D. (1998) How cells respond to interferons. Annu. Rev. Biochem. **67**, 227–264
14 Platanias, L. C. and Fish, E. N. (1999) Signalling pathways activated by interferons. Exp. Hematol. **27**, 1583–1592
15 Haque, S. J. and Williams, B. R. (1998) Signal transduction in the interferon system. Semin. Oncol. **25**, 14–22
16 Liu, M. K., Brouwnsey, R. W. and Reiner, N. E. (1994) Gamma interferon induces rapid and coordinate activation of mitogen-activated protein kinase (extracellular signal-regulated kinase) and calcium-independent protein kinase C in human monocytes. Infect. Immun. **62**, 2722–2731
17 Stancato, L. F., Yu, C. R., Petricoin, E. F. and Larner, A. C. (1998) Activation of Raf-1 by interferon gamma and oncostatin M requires expression of the Stat1 transcription factor. J. Biol. Chem. **273**, 18701–18704
18 David, M., Petricoin, E., Benjamin, C., Pine, R., Weber, M. J. and Larner, A. C. (1995) Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins. Science **269**, 1721–1723
19 Uddin, S., Yenush, L., Sun, X. J., Sweet, M. E., White, M. F. and Platanias, L. C. (1995) Interferon-alpha engages the insulin receptor substrate-1 to associate with the phosphatidylinositol 3′-kinase. J. Biol. Chem. **270**, 15938–15941
20 Burfoot, M. S., Rogers, N. C., Walting, D., Smith, J. M., Pons, S., Paonessaw, G., Pellegrini, S., White, M. F. and Kerr, I. M. (1997) Janus kinase-dependent activation of insulin receptor substrate 1 in response to interleukin-4, oncostatin M, and the interferons. J. Biol. Chem. **272**, 24183–24190
21 Durbin, J. E., Hackenmiller, R., Simon, M. C. and Levy, D. E. (1996) Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. Cell **84**, 443–450
22 Meraz, M, A,, Whitem, J. M., Sheehan, K. C., Bach, E. A., Rodig, S. J., Dighe, A. S., Kaplan, D. H., Riley, J. K., Greenlund, A. C., Campbell, D. et al. (1996) Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signalling pathway. Cell **84**, 431–442
23 Gao, B., Jiang, L. and Kunos, G. (1996) Transcriptional regulation of alpha(1b)adrenergic receptors [alpha(1b)AR] by nuclear factor 1 (NF1): a decline in the concentration of NF1 correlates with the downregulation of alpha(1b)AR gene expression in regenerating liver. Mol. Cell. Biol. **16**, 5997–6008
24 Nguyen, V. T. and Gao, B. (1999) Cross-talk between α_{1B}-adrenergic receptor (α_{1B}AR) and interleukin-6 (IL-6) signalling pathways. Activation of α_{1B}AR inhibits IL-6-activated STAT3 in hepatic cells by a p42/44 mitogen-activated protein kinase-dependent mechanism. J. Biol. Chem. **274**, 35492–35498
25 Chen, J. P., Bao, H. F., Sawyer, S., Kunos, G. and Gao, B. (1997) Effects of short and long term ethanol on the activation of signal transducer and activator transcription factor 3 in normal and regenerating liver. Biochem. Biophys. Res. Commun. **239**, 666–669
26 Kraft, A. S. and Anderson, W. B. (1983) Characterization of cytosolic calcium-activated phospholipid-dependent protein kinase activity in embryonal carcinoma cells. Effect of retinoic acid-induced differentiation of F9 cells to parietal endoderm. J. Biol. Chem. **258**, 9178–9183
27 Roivainen, R., Hundle, B. and Messing, O. R. (1995) Ethanol enhances growth factor activation of mitogen-activated protein kinases by a protein kinase C-dependent mechanism. Proc. Natl. Acad. Sci. U.S.A. **92**, 1891–1895
28 Higashi, K. and Hoek, J. B. (1991) Ethanol causes desensitization of receptor-mediated phospholipase C activation in isolated hepatocytes. J. Biol. Chem. **266**, 2178–2190
29 Starr, R. and Hilton, D. J. (1999) Negative regulation of the JAK/STAT pathway. Bioessays **47**, 47–52
30 Yoshimura, A. (1998) The CIS family: negative regulators of JAK-STAT signalling. Cytokine Growth Factor Rev. **9**, 197–204
31 Hilton, D. J. (1999) Negative regulators of cytokine signal transduction. Cell. Mol. Life. Sci. **55**, 1568–1577
32 Schindler, C., Fu, X. Y., Improta, T., Aebersold, R. and Darnel, Jr., J. E. (1992) Proteins of transcription factor ISGF-3: one gene encodes the 91-and 84-kDa ISGF-3 proteins that are activated by interferon alpha. Proc. Natl. Acad. Sci. U.S.A. **89**, 7386–7389
33 Shuai, K., Schindler, C., Preioso, V. R. and Darnell, Jr., J. E. (1992) A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma. Science **258**, 1808–1812
34 Stephens, J. M., Lumpkin, S. J. and Fishman, J. B. (1998) Activation of signal transducers and activators of transcription 1 and 3 by leukemia inhibitory factor, oncostatin-M, and interferon-gamma in adipocytes. J. Biol. Chem. **273**, 31408–31416
35 Meinke, A., Barahmand-pour, F., Wohrl, S., Stoiber, D. and Decker, T. (1996) Activation of different Stat5 isoforms contributes to cell-type-restricted signalling in response to interferons. Mol. Cell. Biol. **16**, 6937–6944
36 Chung, J., Uchida, E., Grammer, T. C. and Blenis, J. (1997) STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. Mol. Cell. Biol. **17**, 6508–6516

37 Sengupta, T. K., Talbot, E. S., Scherle, P. A. and Ivashkiv, L. B. (1998) Rapid inhibition of interleukin-6 signalling and Stat3 activation mediated by mitogen-activated protein kinases. Proc. Natl. Acad. Sci. U.S.A. **95**, 11107–11112
38 Chen, J. P., Kunos, G. and Gao, B. (1999) Ethanol rapidly inhibits IL-6-activated STAT3 and C/EBP mRNA expression in freshly isolated rat hepatocytes. FEBS Lett. **457**, 162–168
39 Sasaki, Y. and Wands, J. R. (1994) Ethanol impairs insulin receptor substrate-1 mediated signal transduction during rat liver regeneration. Biochem. Biophys. Res. Commun. **199**, 403–409
40 Xu, Y. Y., Bhavani, K., Wands, J. R. and de la Monte, S. M. (1995) Ethanol inhibits insulin receptor substrate-1 tyrosine phosphorylation and insulin-stimulated neuronal thread protein gene expression. Biochem. J. **310**, 125–132
41 Resnicoff, M., Rubini, M., Baserga, R. and Rubin, R. (1994) Ethanol inhibits insulin-like growth factor-1-mediated signalling and proliferation of C6 rat glioblastoma cells. Lab. Invest. **71**, 657–662
42 Mohr, L., Tanaka, S. and Wands, J. R. (1998) Ethanol inhibits hepatocyte proliferation in insulin receptor substrate 1 transgenic mice. Gastroenterology **115**, 1558–1565

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